

EXHIBIT 1



SHORT REPORT

Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells

Atsushi Yawata¹, Masaaki Adachi¹, Hiroyuki Okuda¹, Yasuyoshi Naishiro¹, Takenori Takamura¹, Masato Hareyama¹, Shinichi Takayama², John C Reed² and Kohzoh Imai¹

¹The First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo 060 Japan; ²The Burnham Institute, Cancer Research Center, 10901 N Torrey Pine Road, La Jolla, California 92037, USA

Bcl-2 and a Bcl-2-binding protein BAG-1 function in protection from apoptosis induced by a variety of stimuli. Deregulated expression of Bcl-2 leads to inhibition of apoptosis and is correlated with development of various cancers. Here, we provide evidence that prolonged cell survival introduced by overproduction of Bcl-2 or BAG-1 strongly enhances peritoneal dissemination of human gastric cancer MKN74 cells. Gene transfer-mediated overexpression of Bcl-2 or BAG-1 led to prolonged cell survival of MKN74 cells against serum-starved apoptosis and anoikis. When the viable transfectants were inoculated into the intraperitoneal cavity of BALB/c nude mice, the Bcl-2-expressing MKN74 cells and the BAG-1-expressing MKN74 cells exhibited strongly enhanced peritoneal dissemination in BALB/c nude mice and whole disseminated tumor weights were increased by 4-fold and 3.3-fold, respectively, compared with the control transfectants. The enhanced peritoneal dissemination of MKN74-Bcl-2 and MKN74-BAG-1 transfectants correlated well with resistance to cell death induced by serum-starvation and anoikis. However, the overexpression of Bcl-2 or BAG-1 caused no significant difference among the transfectants in cell growth rates, either *in vitro* or *in vivo*. Taken together, these studies demonstrate that resistance to apoptosis is a crucial factor for development of peritoneal dissemination of human gastric cancer cells.

Keywords: BCL-2; BAG-1; gastric adenocarcinoma; peritoneal dissemination

Apoptosis plays a crucial role in the regulation of embryogenesis, organ development, immune system, repertoire selection and tissue homeostasis. Since apoptosis is responsible for removal of cells that are no longer necessary or dangerous to the host, disturbance of physiological apoptosis may lead to the abnormal cell growth and thus is likely to be associated with tumor progression in a variety of malignancies. Bcl-2 is one of the key molecules for inhibiting apoptosis (Reed, 1993). It has been demonstrated that Bcl-2 is aberrantly expressed in a wide variety of malignant tumors, such as lung and prostate cancers, follicular lymphoma and chronic lymphocytic leukemia (Pezzella *et al.*, 1993; McDonnell *et al.*, 1992; Tsujimoto

and Croce 1986; Adachi *et al.*, 1990). These findings strongly suggest that aberrant Bcl-2 expression may be an important factor for tumorigenesis. Several Bcl-2 homolog and Bcl-2-binding proteins have been identified which participate in either protection from or promotion of apoptosis. Bcl-2 family proteins share at least one of several conserved regions BH1 to BH4. Many but not all Bcl-2 family proteins can interact with themselves or other members of the family (Zha *et al.*, 1996). A Bcl-2-binding protein BAG-1 has been identified. BAG-1 prolongs cell survival in concert with Bcl-2 (Takayama *et al.*, 1995). Since BAG-1 has no conserved regions BH1 to BH4, it is not a member of the Bcl-2 family and may have a unique function in protection from apoptosis.

Peritoneal dissemination is the most common form of recurrence after surgical total resection of many primary cancers, such as gastric, ovarian and pancreatic adenocarcinoma. Thus, the prevention and treatment for peritoneal dissemination are crucial therapeutic targets for these cancers. To prevent this type of recurrence, several efforts have been made. For example, intra-abdominal administration of anti-cancer drugs and abdominal hyperthermia. However, peritoneal dissemination of these cancer cells still remains the most formidable obstacles in their therapy. Although reduction of peritoneal dissemination is essential to improve postoperative survival, few studies have been reported about the process of peritoneal dissemination.

During the process of peritoneal dissemination, gastric cancer cells detach from their primary locations and thereafter survive without adequate interactions between their surface molecules and extracellular matrix proteins. Apoptosis caused by detachment of epithelial cells has been termed 'anoikis' (Frisch and Francis, 1994). Since overexpression of Bcl-2 is known to protect epithelial cells against anoikis, levels of Bcl-2 expression may be a crucial factor for peritoneal dissemination of gastric cancer cells. Though BAG-1 was first discovered because of the ability to associate with Bcl-2, recent study reveals that BAG-1 can also physically associate with hepatocyte growth factor receptors (HGFR) and enhance transduction of HGFR-mediated signals that promote cell survival. Since HGFR is highly expressed in most gastric cancers (Bardelli *et al.*, 1996; Di Renzo *et al.*, 1991), the interaction of BAG-1 with HGFR may be relevant to the progression of these tumors. These accumulating data strongly suggest that overexpression of Bcl-2 or BAG-1 may affect metastasis. In this regard, we have demonstrated previously that overexpression of Bcl-2 or BAG-1 leads to prolonged cell survival of murine

melanoma B16 cells and this enhanced anti-cell death activity promotes their pulmonary metastasis (Takaoka *et al.*, 1997). We report here that overexpression of Bcl-2 or BAG-1 enhances peritoneal dissemination of human gastric cancer MKN74 cells. This increased dissemination potential is associated neither with altered cell morphology nor with enhanced cellular growth, but is well correlated with the ability of Bcl-2 or BAG-1 to protect these cells from anoikis. Thus, our data indicate a linkage of prolonged survival of gastric cancer cells in the absence of cell attachment to extracellular matrix proteins with their ability to disseminate intraperitoneally.

Expression of Bcl-2 and BAG-1 in human cancer cell lines

Overexpression of Bcl-2 is predominantly detected in early-stage rather than advanced-stage gastric adenocarcinomas (Bronner *et al.*, 1995). BAG-1 is ubiqui-

tously expressed in a variety of normal cell types (Takayama *et al.*, 1995), but its expression in gastric cancer has not been explored. We thus investigated expression of Bcl-2 and BAG-1 proteins in various human adenocarcinoma cell lines. Human gastric cancer MKN45 cells contained relatively high levels of Bcl-2 proteins (Figure 1a). BAG-1 was clearly detectable in all cell lines examined, with MKN45 cells showing the highest expression levels (Figure 1a). Thus, cooperative expression of Bcl-2 and BAG-1 is observed in a gastric cancer cell line MKN45, which is consistent with the previous data showing the cooperative *bcl-2* and *bag-1* mRNAs expression in hematopoietic cells (Adachi *et al.*, 1996).

Increased apoptosis resistance of MKN74 cells expressing Bcl-2 or BAG-1

The well-differentiated gastric cancer cell line MKN74 expressed the lowest expression levels of Bcl-2 and

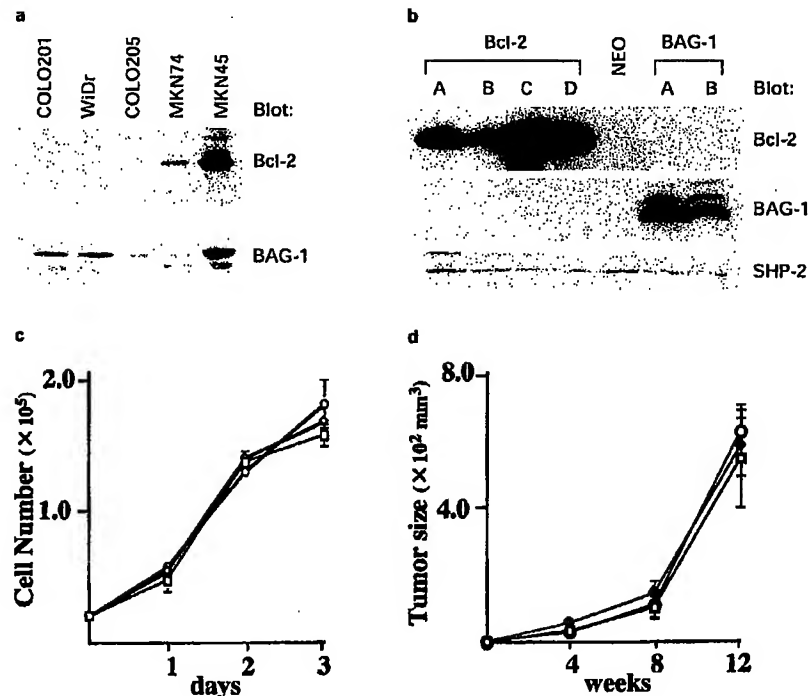


Figure 1 Immunoblot analysis and cell growth of Bcl-2-expressing or BAG-1-expressing MKN74 cells. (a) Cell lysates (50 μ g/lane) from various human cancer cell lines were subjected to SDS-PAGE/immunoblotting using an antibody specific for Bcl-2 (DAKO) or BAG-1 as described previously (Takaoka *et al.*, 1997). Human gastric cancer cell lines MKN74 and MKN45, as well as human colon cancer cell lines COLO201, WiDr and COLO205 cells were provided from Japanese Cancer Research Resources Bank Corp (Tokyo, Japan). MKN45 and MKN74 cells were from poorly differentiated and well-differentiated types of gastric cancers, respectively (Asao *et al.*, 1994). The blots were developed by a standard enhanced chemiluminescence (ECL) method (Amersham). (b) Cell lysates (50 μ g/lane) from the MKN74 cells transfected with *bcl-2*, *bag-1* cDNA or the pcDNA3 plasmid alone (NEO) were subjected to SDS-PAGE/immunoblotting with anti-Bcl-2 or anti-BAG-1 antibodies. The full-length human *bcl-2* cDNA pB4 (Tsujimoto and Croce, 1986), kindly obtained from Dr Y Tsujimoto (Osaka University, Japan), and the full-length murine *bag-1* cDNA (Takayama *et al.*, 1995) were cloned into the eukaryotic expression pcDNA3 vector (Invitrogen) and were transfected into MKN74 cells using Lipofection technique (BRL). The filter was stripped and reprobed with anti-SHP-2 antibody to verify loading of equal amounts of protein. (c) *In vitro* cell growth of the transfectants; 0.25×10^5 viable transfectants were cultured for the indicated times. The culture cells were trypsinized and the viable cell numbers were counted. (d) *In vivo* growth rates of the transfectants; 10^6 viable transfectants in 0.5 ml Hank's balanced salt solution (HBSS; GIBCO BRL) were inoculated subcutaneously into BALB/c nude mice ($n=5$). Tumor sizes were measured as their calculated volumes $[(1/2) \times (\text{longest diameter}) \times (\text{shortest diameter})^2]$ for the weeks indicated. Data represented the mean \pm s.d. of five independent experiments of MKN74-Bcl-2 (circle), MKN74-BAG-1 (diamond) and MKN74-NEO (square). Using the Student's *t*-test, no significant differences were seen among the transfectants for both *in vitro* and *in vivo* cell growth rates.

BAG-1 (Figure 1a) therefore was employed to investigate the effect of overexpression of Bcl-2 or BAG-1. For these experiments, *bcl-2* and *bag-1* cDNAs encoding either full-length Bcl-2 (Tsujiimoto and Croce, 1986) or BAG-1 (Takayama *et al.*, 1995) proteins were stably introduced into MKN74 cells. These gastric cancer cells are capable of dissemination in the murine peritoneal cavity (Asao *et al.*, 1994). After selection in neomycin, the Bcl-2 or BAG-1 transfectant cells were analysed by immunoblotting using Bcl-2- and BAG-1-specific antibodies. Elevated levels of Bcl-2 protein were detected in the MKN74-Bcl-2 transfectants but not in the MKN74-NEO transfectants that received the pcDNA3 parental control plasmid alone (Figure 1b). Although BAG-1 expression was detectable in MKN74 parental cells, markedly elevated levels of BAG-1 protein were found in the MKN74-BAG-1 transfectants as compared with the MKN74-NEO transfectants (Figure 1b). We chose the MKN74-Bcl-2 (clone A) and the MKN74-BAG-1 (clone A) as representative transfectants overexpressing either Bcl-2 and BAG-1, respectively.

We explored the ability of Bcl-2 or BAG-1 to drive cell proliferation in MKN74 cells. When the transfectants were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), their growth rates were not significantly different (Figure 1c). Thus, overexpression of neither Bcl-2 nor BAG-1 proteins affected proliferation of MKN74 cells *in vitro*. In addition, the transfectants (10^6 cells per mouse) were inoculated subcutaneously into nude mice and their tumor sizes were measured. Similar size tumors were produced by all transfected cell lines (Figure 1d). Thus, overexpression of Bcl-2 or BAG-1 protein did not affect cellular growth of MKN74 cells, either *in vivo* or *in vitro*.

We next analysed the relative sensitivity of the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants to apoptosis induced by serum-starvation. At 2 days after serum starvation, loss of cell viability (% of total cells) in the MKN74-NEO, MKN74-BAG-1 and MKN74-Bcl-2 was $59 \pm 5.1\%$, $33 \pm 2.4\%$ and $29 \pm 5.8\%$, respectively (Figure 2a). Thus, overexpression of Bcl-2 or BAG-1 led to resistance to cell death induced by serum starvation. We further examined their DNA fragmentation reflecting cleavage of internucleosomal sites, which is generally associated with the apoptotic process. DNA from the MKN74-NEO transfectants cultured without FBS for 2 days exhibited significant amounts of fragmentation (Figure 2b). In contrast, far less DNA fragmentation was observed in serum-starved cultures of the MKN74-Bcl-2 and MKN74-BAG-1 transfectants (Figure 2b). From these experiments, overexpression of Bcl-2 or BAG-1 in MKN74 cells increased resistance to apoptosis induced by serum starvation.

Bcl-2 and BAG-1 increase resistance to anoikis

After inoculation into the murine peritoneal cavity, cancer cells must survive without adequate cell-matrix or cell-cell interactions. We thus examined the survival of the transfectants under anoikis conditions. The transfectants were counted and plated onto 100 mm petri-dishes, which had been coated with polyHEMA. They could no longer attach the plate and their

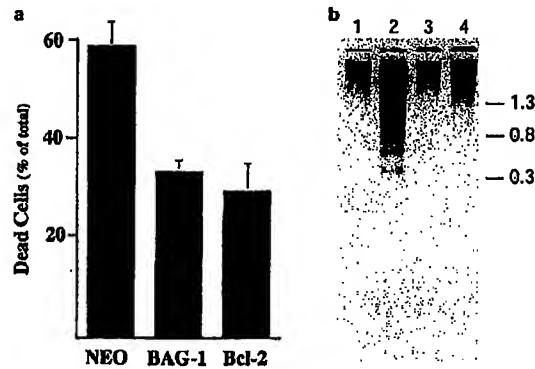


Figure 2 Resistance of transfectants against serum starvation-induced apoptosis. (a) Superior resistance to serum starvation in the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants; transfectants were cultured for 2 days at 5×10^5 cells/ml without FBS. Dead cells were assessed by the loss of ability of the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants to exclude trypan blue. Data represented the mean \pm s.d. of three independent experiments. (b) DNA fragmentation in the serum-starved MKN74 transfectants. The transfectants were seeded at 4×10^6 cells per 100 mm plate without FBS and cultured for 2 days. After washing with PBS, their low molecular weight genomic DNA was extracted with 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris, pH 7.4 at 4°C for 10 min. The DNA was treated with 400 μ g/ml of RNase A for 1 h at 37°C, incubated with 400 μ g/ml of Proteinase K for 1 h at 37°C, ethanol precipitated and analysed (Frisch and Francis, 1994). DNAs from the proliferating MKN74-NEO (lane 1), the starved MKN74-NEO (lane 2), the starved MKN74-BAG-1 (lane 3) and the starved MKN74-Bcl-2 (lane 4) transfectants were loaded into 1.2% agarose gels which were stained with 1 μ g/ml of ethidium bromide and photographed under U.V. light. The size markers to the right were from Φ X174 DNA/HaeIII digested DNA (kb)

viability gradually reduced after 6 h culture under anoikis condition (Figure 3a). However, overexpression of Bcl-2 or BAG-1 clearly reduced loss of cell viability. When 24 h culture under anoikis condition, the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants exhibited prolonged cell survival compared with the MKN74-NEO transfectants (approximately twofold and 1.6-fold increase, respectively) (Figure 3), suggesting that these transfectants had an enhanced ability to survive without adequate cell-matrix interactions. The prolonged cell survival of unattached cells substantially reduced DNA fragmentation in the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants compared with the MKN74-NEO transfectants (Figure 3c). To confirm whether the loss of cell viability was due to anoikis, we analysed the effect of β 1 integrin (CD29)-mediated signaling using anti- β 1 antibody K20. The anti- β 1 antibody K20 stimulates tyrosine phosphorylation of proteins of 115 to 130 kDa in an epidermal carcinoma cell line (Kornberg *et al.*, 1991). Since this signal mimics the attachment of the cells via β 1 integrin to fibronectin, anti- β 1 integrin antibody-mediated stimulation may suppress anoikis. In all MKN74 transfectants, addition of K20 greatly suppressed loss of cell viability induced by detachment, allowing their cell death to reduce approximately 60% of control transfectants (Figure 3b). This implies that the loss of cell viability induced by detachment can be rescued by integrin β 1-mediated signaling, though its effect is

partial, indicating that their cell death is due to anoikis. Since the inhibitory effects of K20 against their anoikis showed no significant difference among the transfectants (Figure 3b), Bcl-2 or BAG-1-mediated anti-anoikis activity seems not to be directly associated with integrin $\beta 1$ -mediated signaling. However, it is still possible that overexpression of Bcl-2 or BAG-1 may enhance $\beta 1$ integrin-mediated signaling though unlikely.

Peritoneal dissemination of MKN74 transfectants

To explore the correlation of anti-cell death activity with peritoneal dissemination of MKN74 cells, the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants were inoculated into abdominal cavities of nude mice. Six weeks after the inoculation, the MKN74-Bcl-2 and MKN74-BAG-1 transfectants produced more extensive peritoneal metastatic lesions than the MKN74-NEO transfectants. Metastatic lesions exhibited extensive involvement of moderately differentiated adenocarcinoma from MKN74 transfectants, and histological sections of a representative tumor from the MKN74-Bcl-2 transfectants are shown in Figure 4a and b. There was no significant difference of pathological findings among the transfectants (data not shown). We next evaluated their dissemination potential by comparison of the whole tumor weights in the murine peritoneal cavity. The MKN74-Bcl-2 and MKN74-BAG-1 transfectants showed clearly elevated peritoneal dissemination by fourfold (4.0 ± 0.5) and 3.3-fold (3.3 ± 0.6) higher weights, respectively compared with the MKN74-NEO transfectants (Figure 4c). Other experiments using other independently derived MKN74-Bcl-2 and MKN74-BAG-1 over-expressing cell lines produced similar results; the MKN74-Bcl-2 transfectants (clone C in Figure 1b) and MKN74-

BAG-1 transfectants (clone B) showed clearly elevated peritoneal dissemination with fourfold (4.0 ± 0.6) and 3.6-fold (3.6 ± 0.5) higher weights, compared with the MKN74-NEO transfectants (data not shown).

In the present data, we provide evidence that gene transfer-mediated overexpression of Bcl-2 or BAG-1 promotes peritoneal dissemination of a human gastric cancer cell line MKN74. Theoretically, this promotion of peritoneal dissemination of gastric cancer cells might be caused by biological activities related to overexpression of Bcl-2 or BAG-1 protein other than their anti-cell death activity. However, these transfectants exhibited similar growth rates and produced subcutaneous tumors with similar sizes compared to MKN74-NEO control cells. In addition, their cellular morphology was not significantly different. We thus exclude possibilities that the promotion of peritoneal dissemination by overexpression of Bcl-2 or BAG-1 proteins was due to the enhanced proliferation or altered expression of cell surface molecules may affect cell-cell interactions. Overexpression of two different apoptosis antagonists Bcl-2 and BAG-1 similarly enhanced peritoneal dissemination (Figure 4), and MKN45 cells expressing high levels of Bcl-2 and BAG-1 (Figure 1) exhibited significantly larger amount of peritoneal dissemination in nude mice than MKN74 cells (Asao *et al.*, 1995). These data suggest that prolonged cell survival can promote peritoneal dissemination of MKN74 cells. When combined with our previous data demonstrating that overexpression of Bcl-2 or BAG-1 in murine malignant melanoma cells leads to enhanced pulmonary metastasis (Takaoka *et al.*, 1997), our data strongly suggest that resistance to apoptosis is a crucial factor for tumor cell metastasis in a wide variety of malignancies.

To accomplish peritoneal dissemination, cancer cells must survive without cell-cell interactions or cell-matrix

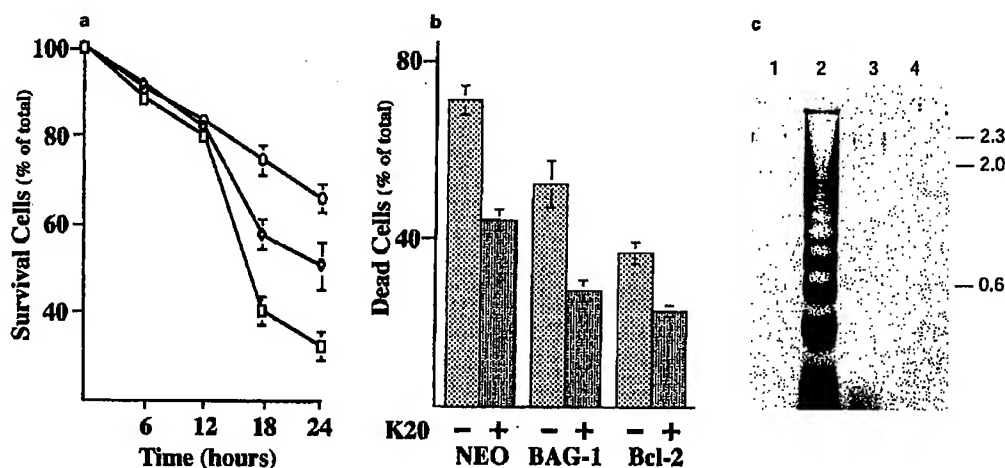


Figure 3 Resistance of Bcl-2 and BAG-1 transfectants against anoikis. (a) Transfectants were cultured at 5×10^5 cells/ml in polyHEMA (Aldrich Chemical Co., Milwaukee, WI) coated plates for the indicated times. Cell survival was assessed by trypan blue exclusion assay, for the MKN74-Bcl-2 (circle), MKN74-BAG-1 (diamond) and MKN74-NEO (square) transfectants. (b) Transfectants were cultured at 5×10^5 cells/ml in polyHEMA coated plates for 24 h with (+) or without (-) addition of anti-integrin $\beta 1$ antibody K20 (10 μ g/ml; Coulter) and polyclonal rabbit anti-mouse antibody (20 μ g/ml; Jackson Laboratories). Dead cells were assessed by failure to exclude trypan blue. Data represented the mean \pm s.d. of three independent experiments. (c) DNA fragmentation in the transfectants under anoikis condition. The control MKN74-NEO (lane 1), and the MKN74-NEO (lane 2), MKN74-BAG-1 (lane 3) and MKN74-Bcl-2 (lane 4) transfectants were cultured under anoikis conditions for 24 h and their genomic DNAs were loaded into 1.2% agarose gels. The size markers to the right were from λ phage HindIII-digested DNA (kb)

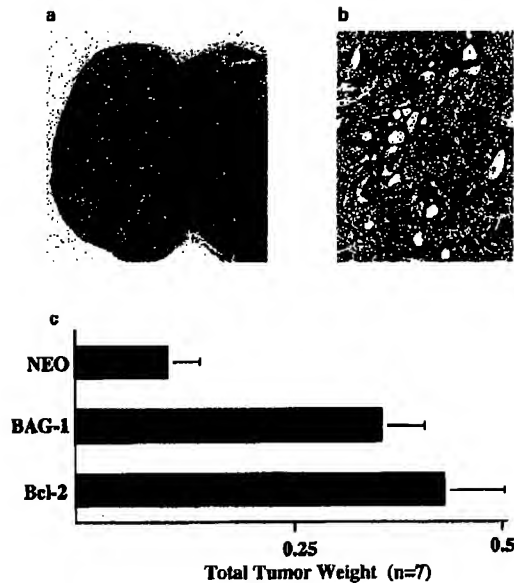


Figure 4 Peritoneal dissemination potential of MKN74-transfectants. (a) Representative whole mount specimen of a tumor in an abdominal cavity where the MKN74-Bcl-2 transfectants were inoculated (original magnification $\times 24$). The tumors were preserved in neutralized 10% formaldehyde solution, subsequently followed by pathological investigation. (b) All tumors were predominantly composed of moderately-differentiated adenocarcinoma (original magnification $\times 240$). (c) Abdominal dissemination potential of the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants was evaluated by increases of tumor weights in the abdominal cavity. Extensive peritoneal metastatic lesions were found in mice that the MKN74-Bcl-2 or MKN74-BAG-1 transfectants were inoculated, whereas far less metastatic lesions were found in mice the MKN74-NEO transfectants were inoculated. The viable MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants (10^5 cells) in 0.5 ml HBSS were injected into the abdominal cavity of nude mice ($n=7$). Six weeks after the inoculation of the transfectants, the mice were sacrificed and examined whether they developed peritoneal dissemination. Bars represent the means \pm s.d. of whole tumor weights in grams ($n=7$ per each transfectant)

interactions prior to adhesion of cancer cells to the murine peritoneal mesothelium. Although several studies indicate that loss of requirements for cell-cell interactions in gastric cancers promotes tumor progression (Oda *et al.*, 1994), we could not find any significant difference in cell aggregation among transfectants (data not shown). To explore possible mechanisms for linking anti-cell death activity and enhanced peritoneal dissemination of the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants, we investigated their cell survival under anoikis conditions. It has been reported that after the inoculation of cancer cells into peritoneal cavity, the first adhesion of cancer cells to the peritoneum takes place on days 5–7, and the cancer cells begin to proliferate and infiltrate the muscle layer on days 9–11 (Buck *et al.*, 1973). This suggests that the MKN74 transfectants must survive for at least 5 days without adequate cell-matrix interactions before they successfully invade the murine

peritoneum. Our anoikis assays strongly suggest that the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants exhibited superior viability under anoikis conditions, in comparison with control transfectants. This implies that the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants can survive for a longer period in the peritoneal cavity than the MKN74-NEO transfectants. The prolonged cell survival under anoikis conditions was well correlated with enhanced peritoneal dissemination of the transfectants (Figures 3 and 4). Thus, it is likely that the MKN74-Bcl-2 and MKN74-BAG-1 transfectants gain enhanced peritoneal dissemination potential by reduced requirement of cell-matrix interaction via prolonged cell survival.

It has been shown that overexpression of Bcl-2 strongly inhibits apoptosis induced by disruption of epithelial cell-matrix interactions (Frisch and Francis, 1994), and that interactions with extracellular matrix suppress apoptosis in mammary epithelial cells (Boudreau *et al.*, 1995). Here, our data clearly demonstrate that overexpression of BAG-1 inhibits anoikis of epithelial cells, in addition to Bcl-2. It is of interest to investigate whether anti-apoptotic molecules generally can inhibit anoikis and whether Bcl-2 or BAG-1 is functionally associated with integrin $\beta 1$ -mediated signaling.

Several studies have examined the levels of Bcl-2 expression in normal gastric epithelial cells and gastric cancers. Bcl-2 protein is highly expressed in the earliest precursor dysplastic lesions of gastrointestinal epithelium, as well as majority of gastric adenocarcinomas (Bronner *et al.*, 1995). Immunohistochemical studies have failed to demonstrate a correlation between Bcl-2 expression and overall patient survival (Lauwers *et al.*, 1995). These findings imply that the detection of Bcl-2 protein alone is not likely to reflect anti-apoptotic activity in the cancer cells, since many other molecules, which function as either inhibitors or promoters of apoptosis, may also play crucial roles in the tumor cells and cooperatively determine their fate. In this context, little information is available that links anti-apoptotic activity to metastatic potential or peritoneal dissemination of gastric cancers. Thus, the data indicating enhancement of peritoneal dissemination of gastric cancer cells by overexpression of either Bcl-2 or BAG-1 proteins reveals a crucial role of anti-apoptotic activity during development of peritoneal dissemination of gastric cancer cells. If we find means which can counteract the effects of anti-apoptotic proteins such as Bcl-2 and BAG-1, these therapies may greatly improve suppression of tumor metastasis.

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